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13. ABSTRACT (Maximum 200 Words)

A strategy to identify mutant genes using inhibition of nonsense mediated decay (NMD) in cell lines has been proposed by others. Blocking translation with antibiotic emetine has been shown to inhibit the NMD. Stabilization of mutant mRNA following the inhibition of NMD with emetine can be detected using microarray technology, such as Affymetrix genechips, for example. Unfortunately, too many genes that do not contain any mutations show mRNA increase following emetine treatment due to stress response to the inhibition of translation or due to being a natural substrate for NMD, thus complicating the identification of mutant genes. We have developed a simple analytical method that increases the efficiency of identifying mutant genes in cell lines following inhibition of nonsense mediated decay (NMD). The approach assumes that the spectra of mutant genes differ between cell lines of the same tumor origin. Using this analytical filter we have identified previously unknown inactivating mutations in the Jak1, Synaptojanin2 (SYNJ2) and Cleft Lip and Palate Transmembrane Protein 1(CLPTM1) genes in the prostate cancer cell line LANCaP. A list of candidate genes for sequencing analysis has been generated for the other prostate cancer cell lines.

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INTRODUCTION

It is widely accepted that cancer is a genetic disease and that inactivation of tumor suppressor genes as well as activation of oncogenes is the causes of cancer. Finding genes responsible for the development of cancer has a diagnostic and prognostic significance and would help to create drugs against the disease. Although for some cancer types a number of genes and pathways for carcinogenesis have been already identified, the mechanisms of prostate cancer development remain poorly understood. Loss of heterozygosity (LOH) studies indicate that there are many yet unknown prostate cancer related tumor suppressor genes. As the title of the review by Ostrander EA and Stanford JL. in the American Journal of Human genetics (2000 pp 1367-75) says: Genetics of Prostate Cancer: Too Many Loci, Too Few Genes. The goal of this grant proposal is using a novel gene identification strategy, called GINI analysis, to identify genes that contain inactivating mutations in both alleles or contain mutation in one and have lost the other allele in prostate cancer cell lines. Such genes, if also mutated in primary tumours, can be putative prostate tumour suppressor genes.

The most frequent gene inactivating mutations are those that result in generation of premature translation termination codons (PTC), that is nonsense or frameshift mutations. PTC if occurs more than 50 bp upstream of the last exon junction initiates rapid degradation of mutant mRNA through the Nonsense Mediated mRNA Decay (NMD) pathway. Blocking translation with antibiotic emetine has been shown to inhibit the NMD. Stabilization of mutant mRNA following the inhibition of NMD with emetine can be detected using microarray technology, such as Affymetrix genechips, for example.

Unfortunately, too many genes that do not contain any mutations show mRNA increase following emetine treatment due to stress response to the inhibition of translation or due to being natural substrate for NMD, which complicates the identification of mutant genes. The goal of this grant proposal is first to develop a strategy to distinguish the mutant mRNA stabilization due to inhibition of NMD from the mRNA increase of non-mutated genes due to stress response to emetine and second to identify mutant genes in prostate cancer cell lines using the inhibition of NMD strategy.

This is the first year report for this grant covering the twelve month period from January 2004 till December 2004. Unfortunately, I could find a postdoctoral researcher to work on this project only from the July of the 2004. However, the tasks of the proposal projected for the first year period has been essentially completed.

BODY:

Specific Aim1. Identification of the candidate tumor suppressor genes for prostate cancer.

Task1. Application of different methods of NMD inhibition to select candidate genes for mutation analysis.

To complete this task we planned:

a) To identify genes that show an mRNA increase following emetine treatment of normal prostate epithelial cells PrEC, b) to optimize the conditions for blocking the expression of hUpf1 and hUpf2 genes using siRNA transfections, c) create cell lines expressing both mutant dominant negative, and wild type, forms of the hUpf1 gene, d) using Affymetrix genechips to identify genes that show mRNA increase following the treatments with 1) emetine, 2) emetine and actinomycin D and 3) by blocking the function of hUpf genes, e) to analyze the results of all Genechip experiments in order to select genes that show consistent mRNA increases following all methods of NMD inhibition.

a) We have identified genes that show mRNA increase following the emetine treatment in the immortalized prostate epithelial cell line RWPE-1. The advantage of using these cells instead of PrEC is that they grow faster and they are easier to handle in tissue culture. These epithelial cells derived from the peripheral zone of a histologically normal adult human prostate were transfected with a single

copy of the human papilloma virus 18 (HPV-18). They, as well as initially planned PrEC, are not supposed to contain mutations in prostate tumour suppressor genes, for this reason, they are as good as PrEC for the control emetine treatment experiments.

b) We tested the feasibility of the siRNA approach to inhibit NMD for the high-throughput identification of mutant genes on the two colon cancer cell lines LS180 and RKO, in which mutated genes has been previously identified. The hUpf1 and hUpf2 are reportedly the key regulators of the NMD [1, 2] and the sequence of the siRNA oligos that downregulate the expression of the hUpf1 and hUpf2 genes resulting to inhibition of NMD in Hela cells have been published previously [1]. We ordered the oligos for the hUpf1 and hUpf2 genes. We treated the RKO and LS180 cells with the different concentrations of double stranded oligos according to the manufacturer instructions. We have found that while the treatment of the RKO cells with the siRNA resulted in the decrease of the mRNA for the hUpf1 and hUpf2 genes, the level of the mRNA for these genes in the LS180 cells remained unchanged (Figure 1).

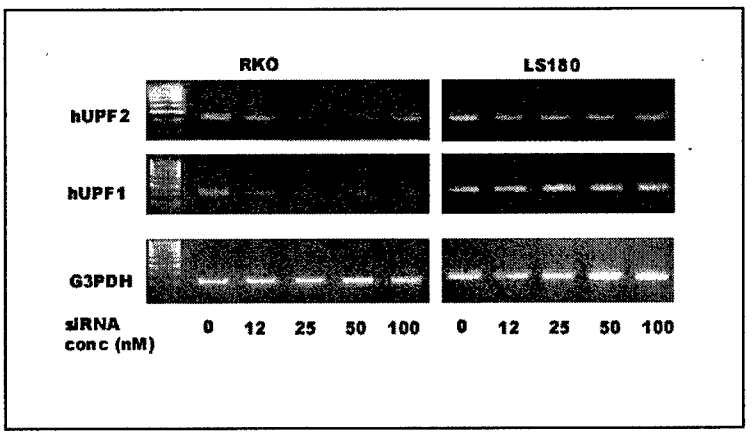

Figure 1. Downnregulation of hUpf1 and hUpf2 mRNA in the RKO but not in LS180 colon cancer cells following transfection with the siRNA oligos directed against hUpf2 (top bands) or hUpf1 (middle bands)

This implies that transient transfections of siRNA oligos work well not for any cell line and that this approach can not be used as a high-throughput method of the identification of mutations. Besides, when we analyzed mRNA changes in the RKO cells following the siRNA treatment for the hUpf1 gene using cDNA microarray we observed that the siRNA-mediated stabilization of the mutant mRNA for the p300 gene is not sufficient to select the gene for sequencing analysis. Recent publication on the inhibition of NMD using siRNA approach in the Hela cells [3] (Hela cells are transfected with siRNA very efficiently) has demonstrated that almost 5% of all genes undergo mRNA increase at least two-fold following hUpf1 downregulation. This suggest that the advantage of using siRNA against hUpf 1 gene versus emetine treatment is questionable, since too many non-mutated genes show mRNA increase following hUpf1 silencing either. We decided not to persuade the siRNA approach further and try to find a more efficient method of distinguishing mutant mRNA stabilization from the mRNA increases for non-mutant genes.

c) We also tested the efficiency of the stable expression of the dominant negative mutant form of the hUpf1gene in the RKO cells on the stabilization of mutant mRNA for the p300 gene. We transfected the RKO cells with the wild type or R844C dominant negative mutant of the hUpf1 gene expression plasmids under CMV promoter kindly provided by Dr. Maquat. We have isolated stable tranfectants and compared the mRNA profiles from the wild type and mutant counterparts. No significant changes in the mRNA level have been observed between mutant and wild type hUpf1 gene expressing clones. This could be due to insufficient level of expression of the dominant negative hUpf1 protein in the stable transfectants to abrogate the function of the endogenous wild type hUpf1. Unfortunately the expression constructs for the wild type or dominant negative forms of the hUpf1 gene provided by Dr. Maquat did not include any tags, therefore we were unable to monitor the level of the exogenous hUpf1 expression. We are working currently on the adenoviral vector constructs to be able to transiently overexpress the

different versions of the dominant negative mutants of the hUpf1 gene. These adenoviral constructs will contain FLAG tag to monitor the level of expression of the hUpf1 gene.

Although we have succeeded in finding mutant genes in the RKO colorectal cancer cell line using emetine and actinomycin D combined treatment, the inhibition of transcription results in the significant decrease of the amount of mRNA. This makes almost halve of all hybridization signals on the Affymetrix genechip undetectable. This implies that too many genes are lost for the analysis when actinomycin D is used and that this reagent is not suitable for the high-throughput analysis.

e) In order to complete the task 1 despite the described difficulties we have developed a simple and robust analytical algorithm which allows efficient selecting of candidate genes for sequencing analysis among thousand of genes that showed mRNA increase following emetine treatment. This algorithm is based on the assumption that different genes are mutated in different prostate cancer cell lines due to the heterogeneity of prostate cancer. The method utilises comparing mRNA profiles of two cancer cell lines before emetine treatment and also comparing between cell lines mRNA changes produced by emetine treatment. Genes selected for sequencing analysis should satisfy the following criteria: 1) the gene should have low expression in one cell lines and the high expression in the other, 2) the genes should show a high mRNA increase following emetine treatment only in cell line with low expression before treatment.

We treated the two prostate cancer cell lines LANCaP and PC3 with emetine, prepared total RNA from emetine treated as well as untreated cells, prepared labelled RNA probes and hybridized it to Affymetrix genechips containing 14,000 genes. We applied our simple algorithm to look for mutant genes in these cell lines. The seven genes for the LNCaP and one gene for the

Table 1. A list of candidate genes in the LNCaP and PC3 selected by comparing the expression profiles of the LNCaP and PC3 cells before and after emetine treatment

Gene Symbol	Gene Title	LNCaP emetine induction Log_2 (fold-change)	PC3 emetine induction Log_2 (fold-change)	LNCaP absolute signal from untreated cells cells cells	PC3 absolute signal from untreated cells cells cells	LNCaP/PC3 untreated Log_2 ratio
RIN2	Ras and Rab interactor 2	3.2	0.3	207	3776	-4.2
JAK1*	**Janus kinase 1**	**2.8**	**0.5**	**477**	**9086**	**-4.3**
NRP1	Neuropilin 1	2.6	-0.2	543	10577	-4.3
CLPTM1	**Cleft lip and palate transmembrane protein 1**	**2.4**	**0.1**	**338**	**4083**	**-3.6**
SYNJ2	**Synaptojanin 2**	**2.2**	**-0.4**	**340**	**6414**	**-4.2**
DATF1	death associated transcription factor 1	2.1	0.7	498	2169	-2.1
RANBP2	RAN binding protein 2	2.1	0.5	517	4063	-3.0
TP53	**Tumor protein p53**	**-0.2**	**2.8**	**13656**	**298.6**	**5.5**

*In **Bold** are the genes with identified bi-allelic mutations

PC3 cells satisfied the parameters of the filter (Table 1and the appended manuscript, which has been accepted to Cancer Genetics and Cytogenetics).. The selected gene for the PC3 cells was p53, which is known to contain nonsense mutation in the PC3. We sequenced the five out of seven genes selected for the LNCaP cells and found bi-allelic inactivating mutations in the three genes. This implies that our algorithm is the most efficient up today for finding mutant genes using inhibition of NMD and microarray analysis. The only publication that described finding mutant genes in prostate cancer cell lines

using inhibition of NMD could demonstrate only one mutant gene in total 3 prostate cancer cell lines analyzed [2]. Our filter found 3 genes in only one cell line analyzed.

Since our algorithm misses the genes that have inactivating mutations in both cell lines we applied the same algorithm to compare the LNCaP cells versus the immortalized normal prostate epithelial cells RWPE1. Ten genes selected include the CLPTM1, JAK1 and DATF1 genes, which also have been selected by comparing LNCaP versus the PC3 cells. Among the remaining seven genes is the KLF6 which is a putative prostate tumor suppressor [4].

Table 2. A list of 10 candidate genes for sequence analysis in the LNCaP cells selected by comparing the expression profiles of the LNCaP and RWPE1 cells before and after emetine treatment

Gene Symbol	Gene Title	RWPE1 emetine induction Log_2 (fold-change)	LNCAP emetine induction Log_2 (fold-change)	RWPE1 absolute signal from untreated cells	LNCaP absolute signal from untreated cells	RWPE1/LNCaP untreated Log_2 ratio
LDH1A3	aldehyde dehydrogenase 1 family, member A3	-0.3	2.3	22631	1266.5	4.1
STK17A	serinethreonine kinase 17a (apoptosis-inducing)	-0.5	1.3	4215.4	908.9	2.2
ANKRD15	ankyrin repeat domain 15	-0.5	1.5	1755.6	546.7	1.7
DATF1	death associated transcription factor 1	-0.2	2.1	1576.2	497.8	1.7
NME4	non-metastatic cells 4, protein expressed in	-0.6	1.6	6615.2	485.3	3.8
Jak1	Janus kinase	0	2.8	1329.8	477.3	1.5
BCAR3	Homo sapiens breast cancer anti-estrogen resistance	-0.2	2.3	1480	427.2	1.8
CLPTM1	cleft lip and palate associated transmembrane protein 1	0.1	2.4	2467.8	338.9	2.9
KLF6	**Kruppel-like factor 6**	**0.1**	**3.9**	**1382.4**	**211.9**	**2.7**
RalGDSAF-6	ras association domain containing protein	-0.1	3.2	3565.6	207.7	4.1

In **bold** is the putative prostate tumor suppressor

We also applied our analytical filter to the prostate cancer cell lines 22RV-1 and DU-145. Ten genes for each cell line have been selected for sequencing analysis.(Table 3 and Table 4). The list of genes together with unknown genes includes already known putative prostate tumour suppressor genes as well as genes known to contain inactivating mutations in these cell lines. For example genes selected for the DU145 cells include hMLH1 and SMARCA 4 which are known to contain mutations in the DU145 cells. Sequencing of the Aprin gene in the 22RV1 cells identified inactivating mutations in both alleles.

Table 3. A list of 10 candidate genes for the 22RV1 prostate cancer cells selected by comparing the expression profiles of the 22RV1 and DU145 cells before and after emetine treatment

Gene Symbol	Gene Title	22RV-1 emetine induction Log_2 (fold-change)	DU-145 emetine induction Log_2 (fold-change)	22RV-1 absolute signal from untreated cells	DU145 absolute signal from untreated cells	22RV1/DU-145 untreated Log_2 ratio
TCF8	transcription factor 8	4.6	0.3	293	1616	-1.7
DUSP1	dual specificity phosphatase 1	3.9	1.0	996	5660	-2.3
AS3	**Aprin**	**2.7**	**0.5**	**580**	**1484**	**-1.4**
FLJ11126	DEAD (Asp-Glu-Ala-As) box polypeptide 19-like	2.7	0	123	1381	-2.9
KLF6*	**Kruppel-like factor 6**	**2.6**	**0.9**	**482**	**2025**	**-1.6**
GRAVIN	A kinase (PRKA)	**2.3**	**0.1**	**645**	**3025**	**-2.1**

	anchor protein (gravin) 12					
CRIM1	cysteine-rich motor neuron 1	2.1	0	408	3485	-2.9
IDN3	Nipped-B homolog	1.9	0.6	276	1125	-2.0
FLJ12895	hypothetical protein FLJ12895	1.6	0.2	230	1070	-1.7
EXT1	**exostoses (multiple) 1**	**1.6**	**0.3**	**233**	**1353**	**-2.5**

in **bold** are the putative prostate tumor suppressor genes [4, 5] or genes with confirmed mutations.

Table 4. A list of 10 candidate genes for the DU145 prostate cancer cells selected by comparing the expression profiles of the 22RV1 and DU145 cells before and after emetine treatment

Gene Symbol	Gene Title	DU-145 emetine induction Log_2 (fold-change)	22RV-1 emetine induction Log_2 (fold-change	DU145 absolute signal from untreated cells	22RV-1 absolute signal from untreated cells	22RV1/DU-145 untreated Log_2 ratio
VDUP1	upregulated by 1,25-dihydroxyvitamin D-3	3.3	1	590	5215	3.1
ZBTB10	zinc finger and BTB domain containing 10	3.1	0.5	239	3220	3.8
PHF3	PHD finger protein 3	2.8	0.7	204	1820	3.2
MARKCS*	myristoylated alanine-rich protein kinase C substrate	2.8	0.7	299	4293	3.8
JMJD3	jumonji domain containing 3	2.5	0.8	714	2523	1.8
KLF7	Kruppel-like factor 7	1.9	0.9	386	928	1.3
hSNF2b	Human mRNA for transcriptional activator	1.7	-0.4	539	3581	2.7
MLH1	**mutL (E. coli) homolog 1**	**1.7**	**0**	**1770**	**5868**	1.7
LOC81691	exonuclease NEF-sp	1.5	0	398	2558	2.7
SMARCA4	**SWISNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4**	**1.4**	**-0.2**	**472**	**4240**	3.2

*MARKCS is frequently mutated in colon cancer cells with microsatellite instability.
In **bold** are the genes known to contain inactivating mutations in the DU145 cells.

The sequencing of other candidate genes is currently in progress. We are also planning to compare the expression profiles of the 22rv1 versus LNCaP, 22rv1 versus PC3 and DU145 versus PC3 and LNCaP. Having found the mutant genes in prostate cancer cell lines makes us to conclude that the Task 1 of the Specific aim 1, to develop a reliable method of finding mutations using inhibition of NMD has been essentially completed.

Specific aim 2. Identification of mutations in genes selected from the Affymetrix Genechip.

Task 2. Mutation analysis of the genes selected by using NMD inhibition and Affymetrix Genechip.

To complete this task we planned to perform hybridizations to Affymetrix Genechips, to prepare cDNA from the RNA of the emetine treated cell lines and to sequence cDNA of the selected genes. This task has been completed and the bi-allelic inactivating mutations in the LNCaP cells have been identified in the Synaptojanin2 (SYNJ2), Cleft Lip and Palate Transmembrane protein1 (CLPTM1) (See Fig. 1 and

the appended manuscript) and Jak1 genes. The inactivating mutations in the Aprin gene in the 22rv1 gene has also been identified. Sequencing of other candidate genes in the 22rv1 and DU145 cells is in progress.

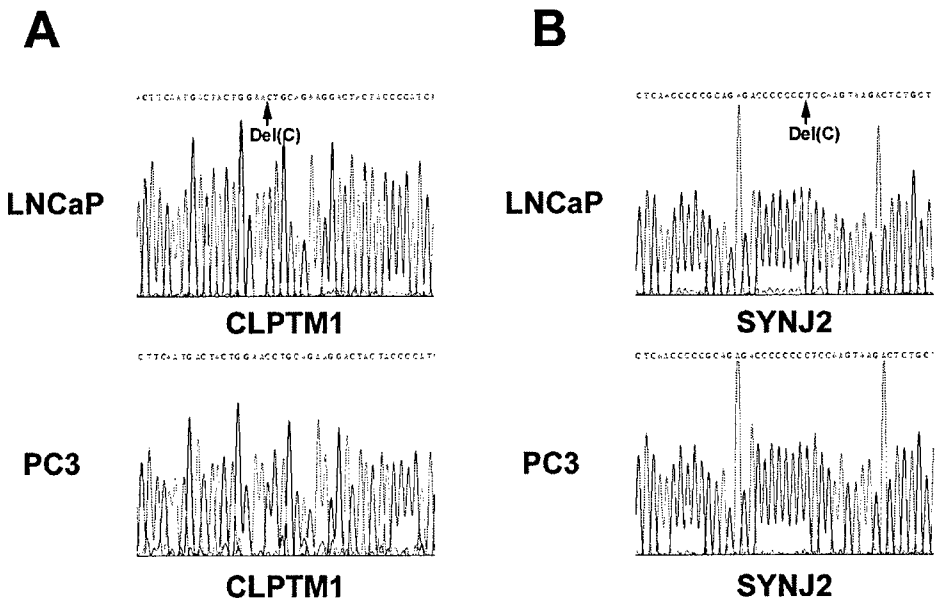

Fig.2 Sequence chromatograms showing frameshift mutation in the CLPTM1 and SYN2 genes in the LNCaP prostate cancer cell line but not in the PC3 cancer cell line.

SUMMARY STATUS OF TASKS OUTLINED IN THE STATEMENT OF WORK

Task1 Complete

Task 2 In progress

KEY RESEARCH ACCOMPLISHMENTS

1) An efficient method of selection of the candidate genes for sequencing analysis from the genes that show mRNA increase following inhibition of NMD with emetine has been developed.
2) A list of candidate genes for sequencing analysis from the LNCaP, PC3, 22RV-1 and DU-145 prostate cancer cell lines has been produced

REPORTABLE OUTCOMES

Publication

Rossi M.R., Hawthorn L., Platt J., Tania Burkhardt T., Cowell J.K and Ionov Y. Identification of inactivating mutations in the JAK1, SYNJ2 and CLPTM1 genes in prostate cancer cells using

inhibition of nonsense mediated decay and microarray analysis. *Accepted to Cancer Genetics and Cytogenetics.*

CONCLUSIONS

Through the funded period of the year 2004 we assessed different approaches to inhibiting NMD in cells to find the best method to distinguish between stabilization of nonsense codon-containing mRNAs from the mRNA increase due to stress response to the agents used to inhibit NMD. We have found that the most efficient approach is to use analytical algorithm, which we have developed to analyse the mRNA changes following emetine treatment as well as mRNA profiles before mRNA treatment in the two or more prostate cancer cell lines in parallel. Using our algorithm we selected a list of candidate genes for sequence analysis for the prostate cancer cell lines LNCaP, 22RV-1, PC3, and DU-145. This list along with the genes unknown to be involved into prostate carcinogenesis includes also the genes that are known to be putative prostate tumor suppressor genes as well as genes known to contain inactivating mutations in the mentioned prostate cancer cell lines. Sequencing five genes from the list from the LNCaP cells identified mutations in the three genes that were not previously known to be mutated in the LNCaP cells. The mutation analysis of the other genes from the list is currently in progress.

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2. Huusko, P., et al., *Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer.* Nat Genet, 2004. **36**(9): p. 979-83.
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4. Narla, G., et al., *KLF6, a candidate tumor suppressor gene mutated in prostate cancer.* Science, 2001. **294**(5551): p. 2563-6.
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Identification of inactivating mutations in the JAK1, SYNJ2 and CLPTM1 genes in prostate cancer cells using inhibition of nonsense mediated decay and microarray analysis.

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ABSTRACT

We have developed a simple analytical method that increases the efficiency of identifying mutant genes in cell lines following inhibition of nonsense mediated decay (NMD). The approach assumes that the spectra of mutant genes differ between cell lines of the same tumor origin. Thus, by analyzing more than one cell line in parallel and taking into account not only changes in mRNA levels following inhibition of NMD, but also comparing mRNA levels between cell lines before the inhibition of NMD, the vast majority of false positives were eliminated from the analysis. In this study we used Affymetrix oligonucleotide arrays to compare mRNA profiles of two prostate cancer cell lines, PC3 and LNCaP, before and after emetine treatment. As a result of our modified approach, from the 14,500 genes present on the array, seven were identified as candidates from LNCaP cells and one was identified from PC3 cells. Sequence analysis of five of these candidate genes identified gene inactivating mutations in four of them. Homozygous mutations were found in the synaptojanin 2 (SYNJ2) and the cleft lip and palate, CLPTM1, gene. Two different heterozygous mutations in the Janus kinase 1 (JAK1) gene result in complete loss of the protein in several different prostate cancer cell lines.

INTRODUCTION

Nonsense mutations that occur in coding DNA sequences upstream of the last exon of a gene elicit a rapid degradation of mutant mRNA through the nonsense mediated mRNA decay (NMD) pathway (Holbrook et al., 2004; Maquat, 2004). Since an initial round of translation is required to initiate degradation of the mutant mRNA, blocking translation with specific drugs, such as emetine, has been shown to abrogate the NMD-mediated degradation of mutant mRNAs (Ishigaki et al., 2001). Inhibition of NMD, therefore, effectively results in an increased amount of mutant cellular mRNA from genes containing nonsense or frameshift mutations which can be detected using expression microarrays. A strategy for using microarray analysis of mRNA profile alterations resulting from inhibiting of NMD in cell lines (GINI) has been proposed for the identification of genes containing nonsense mutations (Noensie & Dietz, 2001). Unfortunately, inhibition of NMD by blocking translation with emetine is accompanied by the upregulation of a large number of genes that do not contain nonsense mutation due to a stress response to the drug, which complicates choosing candidates for sequence analysis. One approach to minimize the number of false positives in the pool of the candidate genes selected for sequence analysis was to combine inhibition of NMD with a block of transcription using actinomycin D. This approach largely prevents de novo synthesis of mRNA during treatment thereby reducing up regulation of stress response genes. Selecting only genes that show mRNA increases following treatment with both emetine and actinomycin D streamlines the mutation analysis. Although this approach has already proved successful in identifying inactivating mutations in colorectal (Ionov et al., 2004) and prostate (Huusko et al., 2004) cancer cell lines, the analysis is

compromised by the overall reduction in mRNA transcripts which results in loss of hybridization signal for almost half of the genes present on the microarray. Thus, genes which may be mutated, but which are expressed at moderate to low levels, are excluded from detection. Although the number of false positive candidate genes could be minimized by using nonmalignant control cell lines to identify those involved in the stress response, the differences in response to drug treatments between normal and cancer cells (Scotto, 2003) still produces a large number of false positives.

We have now developed and tested a simple algorithm to more accurately identify candidate genes for sequence analysis from the large number of genes that show mRNA increases following inhibition of NMD with emetine using prostate cancer cell lines. We used PC3 and LNCaP, both of which reportedly have microsatellite instability (Chen et al., 2003), to maximize the possibility of identifying mutant genes because of their inability to repair replication defects in the coding mononucleotide repeats (Markowitz et al., 1995; Rampino et al., 1997). In this study four of the five candidate genes identified demonstrated either homozygous or heterozygous mutations in a range of prostate cancer cell lines. These findings provide a valuable proof-of-principle that our analytical algorithm can streamline the GINI analysis of cancer cells and identifies novel genes which appear to be related to prostate cancer development and progression.

MATERIALS AND METHODS

Cell culture and emetine treatment. Prostate cancer cell lines LNCaP and PC3 were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. The emetine treatment protocol was as described previously (Ionov et al., 2004).

Oligonucleotide array analysis. mRNA levels in emetine treated and untreated cells were measured using the Affymetrix U133A array. The protocol for this analysis was as outlined in (Kunapuli et al., 2004)

Mutation analysis. One microgram of total RNA from emetine treated LNCaP and PC3 cells was reverse transcribed using the SuperScript II protocol (Invitrogen, Carlsbad, CA). Three overlapping PCR primer sets were used to generate products spanning the entire JAK1 open reading frame (ORF) for both cell types. Genomic DNA isolated from six prostate cell lines, four colon cell lines and ten primary colon tumors was also PCR-amplified using the following JAK1 exon primers: Ex4F 5'-TTCATTTTCCTGCCTTCCAG-3', Ex4R 5'-CCACAAACTCCAGCTTCTCC-3', Ex8F 5' CTGAAGCTCTCTTCCCACGA-3', Ex8R 5'CTAAAACACGGGCTCTCTGC-3'. The primers that amplify the mononucleotide repeat within exon 23 of SYNJ2 in genomic DNA were: SYNJ2 Ex23F 5'-GCC TCC TGT GCT CAG ATC C-3', SYNJ2 Ex23R 5'- GGA GCC GTG TTT TCA GTA GC-3'. The primers to amplify the cDNA containing two short mononucleotide repeats $(C)_5$ of the CLPTM1 gene were: CLPTM1 Ex8-11F 5'- TCA CCA TCA ACA TCG TGG AC-3', CLPTM1 Ex. 8-11R 5'- CGA GGA CTC GAT ATA CGT GGA-3'.

PCR for both cDNA and genomic DNA were performed using the Phusion™ high-fidelity DNA polymerase protocol (Finnzymes OY, Espoo, Finland). The PCR products were gel purified and sequenced using the Applied Biosystems' PRISM 3100 Genetic Analyzer.

Protein analysis. Western blotting was carried out as described previously (Ionov et al., 2004) using a polyclonal JAK1 antibody from Cell Signaling Technology® (Beverly,

MA). The second HRP-anti-rabbit antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Expression Array Analysis. To identify candidate genes related to prostate cancer, we analyzed the mRNA levels between the LNCaP and PC3 prostate cancer cell lines before and after treatment with the NMD inhibiting drug, emetine. Firstly, we excluded genes that were not expressed in either cell line regardless of treatment, as well as genes that were induced by emetine treatment in both cell lines. In the latter case we assumed that the majority of these mRNA increases were most likely due to a cellular stress response related to emetine, rather than inhibition of NMD (see below). We next rationalized that, if we assume that different genes will carry nonsense mutations in different cell lines, then in untreated cells, some genes (those with mutations) would show lower mRNA levels in one cell line (A) and higher mRNAlevels (those without mutation) in the other cell line (B). At the same time only the genes that showed low levels of mRNA should then show increased levels following treatment with emetine. Since it was not possible, however, to predict exactly how a nonsense mutation would affect the RNA levels for any particular gene, we established several arbitrary cut-offs for these mRNA level changes to maximize the chances of identifying mutated genes. Thus, in the comparison between mRNA levels in untreated cells we used the formula $B/A \geq 4$ fold and in the treated cells $A^{treated}/A \geq 4$-fold while for the same gene $B^{treated/}B < 2$-fold. Fold changes are represented by the signal log ratio (SLR) using Affymetrix analysis software (http://www.affymetrix.com/support/technical/manual/expression_manual.affx), and a 4-fold

increase in the absolute signal intensity of emetine treated cells versus untreated cells is equivalent to a SLR = 2 .

Over 800 of the 22,000 probes sets or approximately 3.5% of the total number of genes (14,500) represented on the U133A platform had increased levels of expression following emetine treatment in both LNCaP and PC3 cells. A number of these genes including TXNIP and GADD45B (data not shown) are known stress response genes and were excluded from further analysis. Using the approach described above, we were able to refine our search to seven candidate genes in LNCaP cells (Table 1) as potentially carrying nonsense mutations. RIN2, JAK1, NRP1, CLPTM1, SYNJ2, DATF1 and RANBP2 each had SLR $\geq$ 2 ($A^{treated}/A \geq$ 4-fold) for LNCaP cells and SLR < 1 for PC3 cells ($B^{treated/}B <$ 2-fold). The "p-detection" values for the comparison between emetine treated and untreated LNCaP cells were less than 0.004 for all of these genes. The same approach was used to identify candidate genes in PC3 cells (Table 1). In this analysis we identified p53 as the only candidate mutated gene (p = 0.003). In fact, there have been a number of reports already demonstrating that PC3 cells carry inactivating truncating mutations in p53, whereas LNCaP cells express wild-type p53 (Isaacs et al., 1991; van Bokhoven et al., 2003). During our analysis, we also identified genes whose mRNAs were significantly increased following emetine treatment in both PC3 and LNCaP cells. While these could represent mutant genes in both cell lines, they could equally be stress response genes induced in both cells. As such, we have not pursued these genes further at this time.

Mutation analysis of candidate genes. From the genes shown in Table 1, SYNJ2, CLPTM1 and JAK1 were chosen as candidates for mutation analysis based on the fact

that they contain mononucleotide repeat regions within the open reading frame of the genes. The death associated transcription factor 1 gene (DATF1) was also chosen for mutation analysis because its predicted function implicated it as a potential tumor suppressor gene. A homozygous deletion of one "C" nucleotide in a $(C)_8$ mononucleotide repeat in exon 23 (Figure 1A) was identified in the SYNJ2 gene in LNCaP cells. PC3 cells did not show this mutation. Extending the mutation analysis to other prostate cancer cell lines identified a heterozygous mutation in the same region in exon 23 in LAPC-4 cells. We also sequenced exon 23 in the SW48, HCT116, RKO, LoVo and LS180 colon cancer cell lines which are know to have microsatellite instability (MSI). A heterozygous deletion of one "C" in the same mononucleotide repeat was found in LS180 and LoVo cells.

The CLPTM1 gene contains two $(C)_5$ repeats in its open reading frame but sequencing of the entire cDNA in LNCaP cells identified a homozygous deletion of the "857C" nucleotide which lies outside the $(C)_5$ repeats (Figure 1B). No mutations in the CLPTM1 were found in the PC3 cells. Sequencing the cDNA for the DATF1 gene from emetine-treated LNCaP cells did not identify any mutations.

Sequence analysis of the JAK1 cDNA from the emetine-treated LNCaP cells identified two different heterozygous mutations, one in exon 4 which contains a $(A)_8$ mononucleotide repeat (Figure 2A) and the other in exon 8 which contains a $(C)_7$ tract (Figure 2B). Both are predicted to be inactivating mutations indicating a loss of function for JAK1 in these cells (see below). PC3 carried only the wild-type sequence (data not shown). The JAK1 mutations were confirmed by sequence analysis of the same exons from genomic DNA. Sequence analysis of exon-specific PCR products from all 24 exons

of JAK1 did not identify mutations in PC3 as expected. We next extend the analysis of JAK1 to additional prostate cancer cell lines. DU145 cells did not carry mutations in JAK1 but 22Rv-1 and C42 (a derivative of LNCaP) cells both contained heterozygous frameshift mutations of a single "A" in exon 4 and a single "C" in exon 8, which were identical to those seen in LNCaP cells. LAPC-4 cells also carried a heterozygous mutation in exon 8, but in this case the frameshift mutation was an insertion of a "C" nucleotide. In addition to prostate cancer cell lines, we examined genomic DNA from four MSI (+) colon cell lines as well as ten primary colon tumors with MSI. No mutations were found in JAK1 in either the colon cell lines or the colon tumors (data not shown). These results suggest that mutation of JAK1 is more related to the malignant phenotype in prostate cancer cells rather than a general consequence of MSI.

JAK1 expression in prostate cancer cells. Western blot analysis was performed to investigate the presence or absence of the JAK1 protein in prostate cancer cells. As shown in Figure 3, JAK1 is highly expressed in DU145 cells and present at detectable levels in PC3 cells. Neither of these cell lines carry JAK1 mutations. The JAK1 protein was not detectable in either 22Rv-1, LNCaP or C42 cells, and there was only minimal expression in LAPC-4. These data are consistent with the mutation analysis that predicted bi-allelic inactivating mutations in 22Rv-1, LNCaP and C42, and heterozygous inactivation in LAPC-4.

DISCUSSION

In our previous study (Ionov et al., 2004) we demonstrated the utility of manipulation of the NMD pathway for the identification of potential tumor suppressor genes. The major problem with using emetine to block NMD, however, was the

concomitant up regulation of stress response genes which, despite using actinomycin D to prevent de novo transcription, still led to the identification of a large number of candidate genes, many of which were not found to be mutated. In the present study we designed and tested an algorithm for selecting candidate genes for sequence analysis from among the hundreds of genes that show increased mRNA levels after microarray analysis. Using this approach we greatly reduced the number of genes that invited sequence analysis and, of the five genes selected (including p53), truncating mutations were identified in four of them. This success rate greatly improves the utility of the GINI approach to identify candidate tumor suppressor genes. It is possible that the criteria we established to filter the data are too stringent and so we may have missed some genes that carry truncating mutations in the LNCaP and PC3 cells. It is possible, therefore, that lowering the stringency of the filters will result in the identification of a higher number of candidate mutant genes and these analyses are currently underway. It is also possible that we have missed genes that are mutated in both LNCaP and PC3 cells, since they would have the same profile as stress response genes. By including additional cell lines in this analysis in the future, therefore, it may be possible to identify these mutant genes since it is unlikely that the same genes will be mutated in all cell lines tested.

Surprisingly, the number of candidate tumor suppressor genes identified in the LNCaP cells was much higher than in PC3 cells. One possible explanation for this is the MSI status of these cells. While the MSI+ status of the LNCaP cells has been proved by both by our identification of frameshift mutations in microsatellite repeats in the JAK1 and TGF beta receptor type II gene (data not shown), as well as the reported mutation in the MSH2 mismatch repair gene (Leach et al., 2000), the MSI status in the PC3 cells has

been controversial (Karan et al., 2001). If, in fact, PC3 cells do not have MSI, then it may not be surprising that the number of frameshift mutations identified using GINI analysis in these cells might be less than in the LNCaP cells. Another possibility is that the mutation in the p53 gene in PC3 cells could negate the need for mutations in other weaker tumor suppressor genes.

In this study, we identified inactivating mutations in the JAK1 gene, which appears to be specific to prostate cancer cells rather than being a general consequence of microsatellite instability, since MSI+ colon cancer cells did not show mutations in this gene. There are at least four members of the Janus kinase family of tyrosine kinases (TYK2, JAK1, JAK2, and JAK3) that are, with the exception of JAK3, expressed in a variety of different cell types (Verma et al., 2003). The role of JAK1 in interleukin signaling is well documented, as is its role in malignant cell growth and survival (Leonard & O'Shea, 1998). JAK kinases are involved in a number of signaling pathways that mediate malignant transformation through activating STAT, Bcl-2, PI3 and Src kinases, as well as serving to inhibit p53 dependent cell cycle arrest and apoptosis (Leonard & O'Shea, 1998; Quelle et al., 1998; Verma et al., 2003). There is considerable evidence that JAK1 is generally activated in cancer cells and contributes to driving tumor cell growth in both leukemias and solid tumors (Leonard & O'Shea, 1998; Verma et al., 2003). Consequently, cell growth can be suppressed by treatment with the STAT3/JAK2 inhibitor, tyrphostin AG490 in prostate cancer cells (Jacobberger et al., 1999; Ni et al., 2000). It is unclear, therefore, why JAK1 would be inactivated in a subset of prostate cell lines as opposed to being overexpressed. At this time we can only speculate that JAK1 inactivation confers some selective advantage for certain prostate cancer cells, possibly

by allowing for JAK independent signaling. Clearly, a more detailed examination of primary prostatic tumors will be necessary to determine if JAK1 mutations have clinical consequences. It is also of note that the cell lines that are null for JAK1 expression provide a valuable resource for studying the function of this gene.

We have now been able to extend our previous studies (Ionov et al., 2004) to demonstrate that the manipulation of NMD in conjunction with expression array analysis is a powerful tool for identifying novel gene mutations in cancer cells. We have also demonstrated that comparing the consequences of emetine treatment between cell lines from the same tissue provides a more streamlined approach to the identification of mutant genes because it reduces the background of candidate genes that result from stress responses. We are currently extending our sequence analysis of other candidate genes described in this report to determine their involvement in prostate cancer as well as to determine the effectiveness of our data analysis approach to define genes related to the malignant phenotype.

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FIGURES AND TABLES

Table 1. Candidate genes for mutation analysis in LNCaP and PC3 cells. LNCaP and PC3 cells treated with emetine were analyzed using the Affymetrix GeneChip Human Genome U133A Array. Eight candidate genes met our gene expression criteria (i.e. $A^{treated}/A \geq 4$ fold; $B^{treated}/B < 2$ fold). With the exception of TP53, all of the genes have a significant increase (SLR $\geq$ 2) in gene expression in LNCaP cells following emetine treatment, whereas in PC3 cells gene expression was not significantly enhanced (SLR< 1) or diminished following emetine treatment. The signal $\log_2$ ratio (SLR) is a comparison between signal intensities for emetine treated and untreated cells and the absolute signal intensities for each probe are shown to give context to the SLR values. The letters in parenthesis: A- absent, P- present, I- increase, D- decrease, NC- no change. P- values are also provided for the statistical significance of signal changes following emetine treatment.

Figure 1. Mutation analysis in prostate cancer cells. Disruption of NMD suggested that the CLPTM1 and SYNJ2 genes carried nonsense mutations in LNCaP cells. Sequence analysis identifies homozygous deletions within mononucleotide repeats in the coding regions of both of these genes.

Figure 2. JAK1 gene mutations in prostate cancer cells. Genomic DNA isolated from LNCaP, PC3, 22Rv-1, DU145, LAPC-4 and C42 cells were used to confirm gene mutations in the two exons of JAK1 containing microsatellite regions. **A,** DU145 is representative of the wild-type chromatograph for exon 8 which contains a $(C)_7$ tract. Heterozygous frameshift mutations (arrows) were observed in both 22Rv-1 and LNCaP cells, and an insertion of a single "C" (arrowhead) was identified in one allele of LAPC-

4. **B**, Heterozygous frameshift mutations (arrows) were also observed in exon 4 in 22Rv-1 and LNCaP cells. Loss of a single "A" in a region containing $(A)_8$ was seen in alleles of 22Rv-1 and LNCaP when compared to DU145 cells which contain wild-type JAK1.

Figure 3. JAK1 protein in prostate cancer cells. Western blot analysis using 50 μg of total cell lysates for each of the prostate cancer cell lines shown demonstrated the presence of the 130 kilodalton JAK1 protein in DU145 and PC3 cells. There was a faint band for LAPC-4 cells but the JAK1 protein was below detectable levels in 22Rv-1, LNCaP, and C42 cells. The non-specific approximately 250 kd band acts as a protein loading control in this analysis.

Table 1. Candidate genes for mutation analysis in LNCaP and PC3 cells.

Gene Symbol	Gene Title	Probe Set ID	Cyto	LNCaP				PC3			
				Untreated	Emetine	SLR	P-value	Untreated	Emetine	SLR	P-value
RIN2	Ras and Rab interactor 2	209684_at	20p11.22	207 (A)	1022 (P)	3.2 (I)	0.000027	3776 (P)	5003 (P)	0.3 (I)	0.004073
JAK1	Janus kinase 1	201648_at	1p31.3	477 (P)	4125 (P)	2.8 (I)	0.00002	9086 (P)	11753 (P)	0.5 (I)	0.00002
NRP1	Neuropilin 1	212298_at	10p11.2	543 (P)	4479 (P)	2.6 (I)	0.00002	10577 (P)	9798 (P)	-0.2 (NC)	0.942324
CLPTM1	Cleft lip and palate transmembrane protein 1	211136_s_at	19q13.2	338 (P)	2066 (P)	2.4 (I)	0.00002	4083 (P)	4839 (P)	0.1 (NC)	0.5
SYNJ2	Synaptojanin 2	212828_at	6q25.3	340 (A)	1554 (P)	2.2 (I)	0.000068	6414 (P)	5296 (P)	-0.4 (D)	0.999448
DATF1	death associated transcription factor 1	218325_s_at	20q13.33	498 (P)	2256 (P)	2.1 (I)	0.00002	2169 (P)	3165 (P)	0.7 (I)	0.00002
RANBP2	RAN binding protein 2	201711_x_at	2q12.3	517 (P)	2173 (P)	2.1 (I)	0.00002	4063 (P)	6082 (P)	0.5 (I)	0.00006
TP53	Tumor protein p53	201746_at	17p13.1	13656 (P)	12041 (P)	-0.2 (NC)	0.846768	298.6 (A)	2275 (P)	2.8 (I)	0.000241

Figure 1. CLPTM1 and SYNJ2 gene mutations in prostate cancer cells.

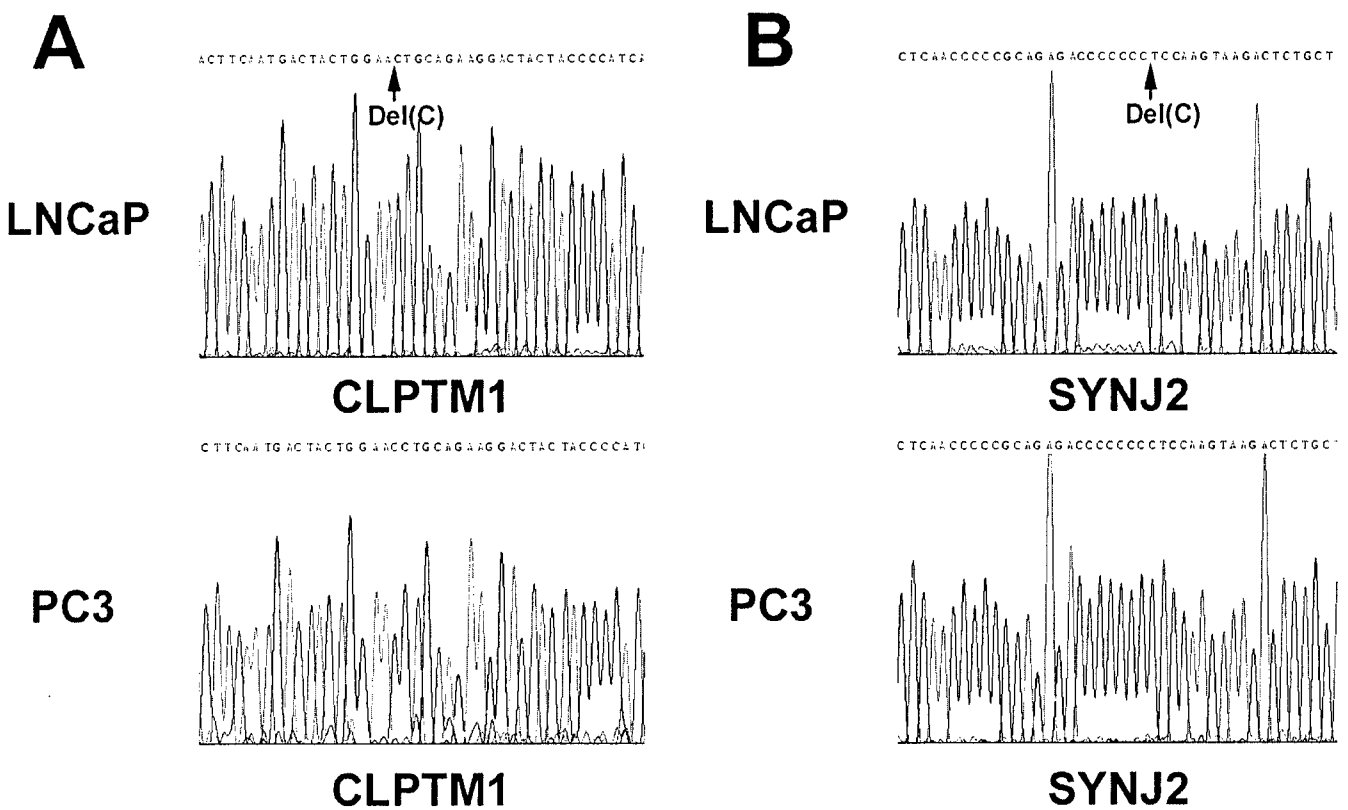

Figure 2. JAK1 gene mutations in prostate cancer cells.

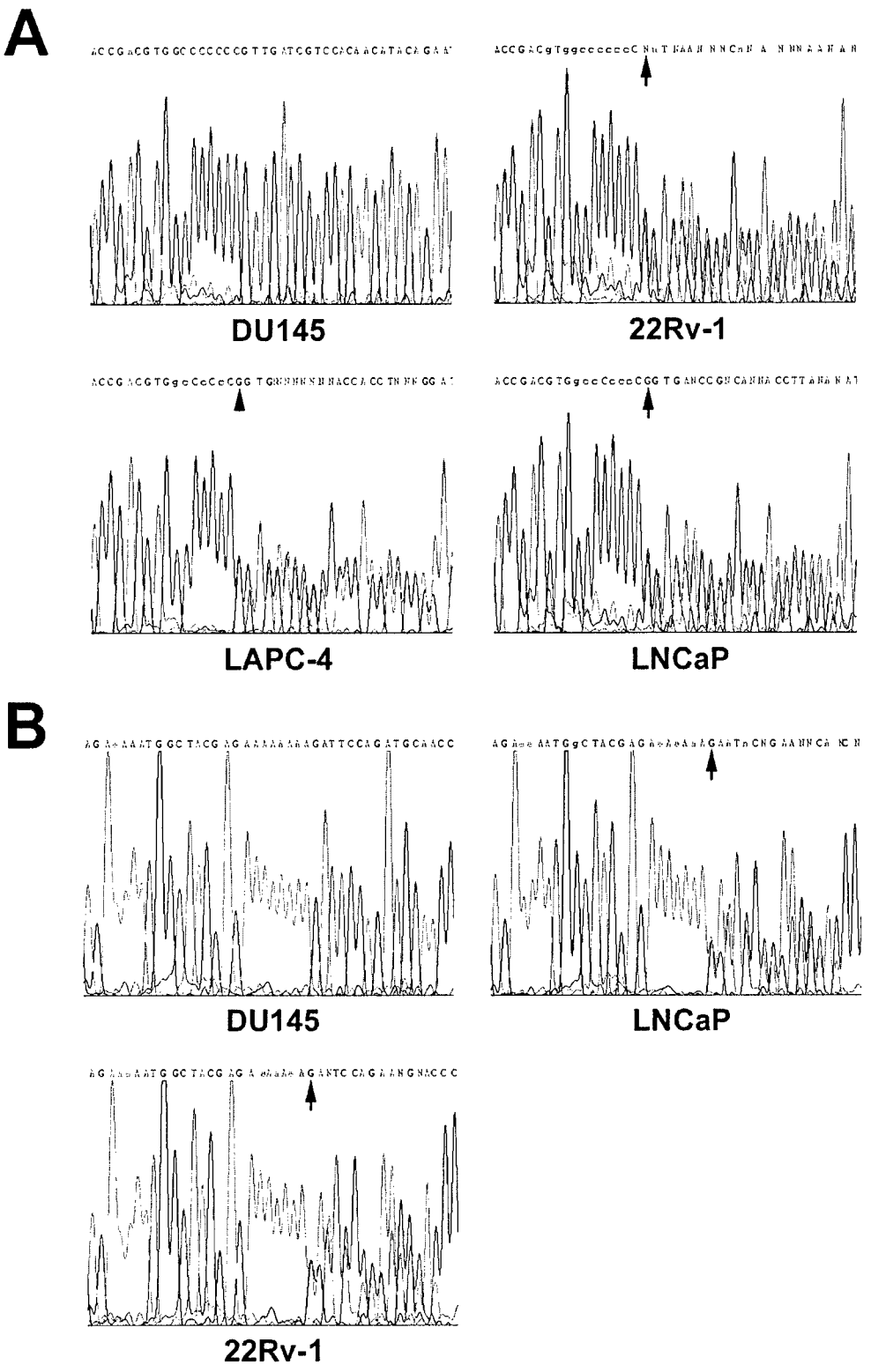

Figure 3. JAK1 protein in prostate cancer cells.